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Effects of temperatures and storage time on resting populations of *Escherichia coli* O157:H7 and *Pseudomonas fluorescens* in vitro☆

O. Modesto Olanya*, Dike O. Ukuku, Brendan A. Niemira

Food Safety and Intervention Technologies Research Unit, U.S. Department of Agriculture, Agriculture Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, PA 19038, United States

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ABSTRACT

Assessment of microbial interactions is crucial for documenting bacterial growth in pure and mixed cultures and their potential for biological applications. *Pseudomonas fluorescens* (non-plant pathogenic and non-pectinolytic) has been used as a biocontrol microbe for plant pathogens and food-borne bacteria. We determined the growth of *Escherichia coli* O157:H7 (*Ec*) and *P. fluorescens*(*Pf*) in monocultures and co-cultures in sterile distilled water (SDW), buffered peptone water (BPW) and trypticase soy broth (TSB). The effects of temperatures (5, 10, 15, 20, 25, 35, and 37 °C) and storage time (0, 2, 4, 6, 24, and 48 h) on bacteria populations were assessed. Bacteria counts in monocultures in SDW ranged from 2.14 to 3.03 and 2.54 to 3.31 Log CFU/ml for *Ec* and *Pf*; respectively. In BPW, mean bacteria counts (monocultures) ranged from 3.15 to 6.14 and 2.54 to 6.41 Log CFU/ml for *Ec* and *Pf*, respectively. *Ec* populations in co-culture varied with storage temperatures and time. After 48 h, *Ec* 43894 monocultures in TSB ranged from 2.17 to 8.75 and 2.31 to 8.85 Log CFU/ml at 20 and 35 °C; respectively. In co-cultures with *Pf* 2-79, *Ec* 43894 counts ranged from 1.71 to 5.83 (20 °C) and 1.90 to 9.03 Log CFU/ml (35 °C) in TSB. The reductions of *Ec* by *Pf* 2-79 varied among strains and generally ranged from 0.20 to 0.90, 0.63 to 1.18, and 0 to 0.56 Log CFU/ml in BPW (10 °C). Substrate availability, storage temperatures, and time significantly ($P < 0.05$) impacted *Ec* populations in co-culture. The liquid substrate experiments indicated suppressive conditions of *Ec* by *Pf*, however; the reduction of produce contamination by *E. coli* O157:H7 during transitory temperature abuse conditions such as the transportation of produce from fields needs further investigation.

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1. Introduction

Escherichia coli O157:H7 and other foodborne pathogens are considered major sources of contamination of leafy greens and other minimally processed vegetables and fruits (Doyle, 1991). The occurrence of pathogenic bacteria on ready to eat vegetables and produce has been extensively documented (Beuchat, 1996). Pathogenic microbes on leafy greens have been primarily controlled by using a combination of physical, chemical, and cultural or sanitary measures (Niemira, Fan, & Sokorai, 2005; Parish et al., 2003). However, the use of competing microbes to improve food safety has gained considerable research attention (Fett, Liao, & Annous, 2011; Leverentz et al., 2006; Ukuku, Zhang, & Huang, 2009).

Interactions of microbial populations such as pathogenic and non-pathogenic bacteria have been the subject of numerous investigations, in an attempt to determine if microbial interactions can lead to detrimental or beneficial outcomes for industrial purposes or for pathogen control (Duffy, Whiting, & Sheridan, 1999; Gombas, 1989; Whipps, 2000). In previously published research, bacterial interactions were assessed to quantify patterns of substrate utilization and microbial growth parameters in nutrient abundant or deficient substrates (Farrag & Martin, 1989; Tranvik & Hofle, 1987). Microbial interactions have also been researched in order to document the behavior of pathogenic and non-pathogenic bacteria and quantify microbial populations. Assessments of bacterial interactions in diverse environments, under limiting and non-limiting conditions have been studied in order to document ecological implications of microbial growth and evaluate potential benefits and industrial applications of bacteria interactions (Tranvik & Hofle, 1987). One of the most practical reasons for quantifying bacterial growth in co-cultures is to determine the potential of microbes for biological control of other pathogenic microbes or detrimental organisms, for industrial uses such as bio-

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* Corresponding author. Tel.: +1 215 233 6471; fax: +1 215 233 6406.
E-mail address: modesto.olanya@ars.usda.gov (O.M. Olanya).

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remediation, and for enhancing the shelf-life of foods in order to reduce spoilage. For example in previous research on competitive growth of food-borne pathogens such as *Listeria monocytogenes* and *Lactococcus lactis* in vegetable broth, it was reported that bacterial growth was limited by the accumulation of lactic acid and the decreased pH (Breidt & Fleming, 1998; Gombas, 1989) due to the growth of other bacteria. The biocontrol of pathogenic microbes in foods by using *Pseudomonas fluorescens*, *Pseudomonas chlororaphis*, and other *Pseudomonas* spp. has been the subject of numerous investigations, perhaps due to the high competitive and antagonistic ability of the bacterium and its effectiveness in various systems (Buchanan & Bagi, 1999; Cheng, Doyle, & Luchansky, 1995; Fett, 2006; McKellar, 2007; Samelis & Sofos, 2002). In other studies, the addition of a compound such as glucose in trypticase soy broth was shown to inhibit *E. coli* O157:H7 by *P. fluorescens* in vitro (Samelis & Sofos, 2002). In a different research, *P. fluorescens* strains in combination with *Bacillus* sp. were effective for biocontrol of *E. coli* O157:H7 on Jalapeno pepper and bell pepper (Liao, Cooke, & Niemira, 2010). Similarly, *P. fluorescens* has been documented in successful biocontrol of various soil-borne plant pathogens mainly of agronomic crops (Weller, 1988). In the above studies, the effectiveness of *P. fluorescens* was attributed to its competitive ability and colonization potential of leaf surfaces and soil rhizospheres in comparison to other microbes. In addition to that, the production of various antibiotics such as phenazines and 1, 4-dicholoroglucanol by many saprophytic strains of *P. fluorescens* has been reported as contributing to its antagonism or suppression of plant pathogens (Weller, 1988).

Microbial interactions have often been evaluated with bacterial strains in incubation studies in monocultures and mixed cultures (co-inoculations). For example, experimental results on the effects of microbial concentrations or diverse cell densities may be used in assessing synergistic or detrimental effects of co-cultures relative to the monoculture. *P. fluorescens* (non-plant pathogenic and non-pectolytic) has been successfully used as biocontrol for plant pathogens (Weller, 1988) and shown to inhibit some food-borne microbes (Buchanan & Bagi, 1999). However, there is limited information on the effects of temperatures and storage times on populations of *P. fluorescens* (non-plant pathogenic and non pectinolytic) and *E. coli* strains in monocultures or interactions between the co-inoculations (mixed cultures).

In previous research, we reported that *P. fluorescens* may show low to moderate suppression of *E. coli* O157:H7 bacteria inoculated on the surface of spinach leaves (Olanya, Annous, Niemira, Ukuku, & Sommers, 2012). In that study we did not investigate the interactions between the native microbiota of spinach and the inoculated human pathogen. The objective of this study was to monitor the behavior of monocultures of *P. fluorescens* and *E. coli* O157:H7 and their interaction as a co-culture in limited nutrient environments. Therefore, sterile distilled water and buffered peptone water (BPW) were used as the substrates to study survival and growth of populations of *P. fluorescens* and *E. coli* O157:H7. The extent to which *P. fluorescens*, may impact survival of *E. coli* O157:H7 in limited nutrient substrate and storage conditions may be used in deducing if microbial competition for substrates is a major component for competitive exclusion between these bacterial populations. In this study, the effect of storage temperatures and time on microbial interactions under diverse conditions was used to assess whether the interactive effects of *P. fluorescens* and *E. coli* O157:H7 are conducive or detrimental to pathogen growth or control based on in-vitro assays. Also, we assessed the effect of relative ratios of *P. fluorescens* to *E. coli* O157:H7 that may impact survival and growth of *E. coli* O157:H7 population.

2. Materials and methods

2.1. Bacterial strains

The bacteria used in this study were *E. coli* O157:H7 strains ATCC 43894, 43895, 35150 obtained from USDA-ARS-ERRC culture collection. In addition, *P. fluorescens* strains 2-79; Q287 and Q8 R1 were obtained from Dr. David Weller (USDA-ARS Root Diseases and Biological Control Unit, Pullman, WA). The latter isolates were originally isolated from soil rhizospheres from fields previously cultivated to wheat (Weller, 1988). The isolates of *P. fluorescens* are non-plant pathogenic and non-pectolytic. All stock cultures were maintained in Tryptic Soy Broth (TSB; BBL, Sparks, MD) containing 20% glycerol at −80 °C. Prior to use in the experiments, a loopful (~0.05 ml) of stock culture was transferred to 4.5 ml TSB and incubated at 37 °C (*E. coli* O157:H7) or 26 °C (*P. fluorescens*) for 24 h. A loopful of this culture subsequently was transferred into fresh 4.5 ml TSB and incubated at the same temperatures for another 24 h. This culture was then spun down at 10,000 rpm for 5 min at 4 °C. The bacterial cell pellet was washed twice with 0.1% buffered peptone water (BPW, BBL), and re-suspended in 4.5 ml of PW. The suspended culture was diluted in BPW to give final cell concentration of approximately 3–5 Log CFU/ml. This culture was used as the inoculum.

2.2. Bacteriological media

Bacteriological media consisting of trypticase soy agar (TSA) and Pseudomonas Agar F (PAF) were used for quantifying cell density of *E. coli* O157:H7 (*Ec*) and *P. fluorescens* (*Pf*); respectively, (Difco, Detroit, MI). Similarly, Restaino and Frampton (R & F) *E. coli* O157:H7 chromogenic plating medium (RFCM), which is specific for isolation and identification of *E. coli* O157:H7, was also used (R & F Products, Inc., Downers Grove, IL) based on standard methods (Restaino, Frampton, Turner, & Allison, 1999). All media and associated supplements were prepared according to the manufacturer's specifications, and pH adjusted to the required level where necessary.

2.3. Preparation of bacterial culture

Serial dilutions of *E. coli* O157:H7 and *P. fluorescens* strains inoculua stated above were performed. *E. coli* O157:H7 suspensions (2 ml) at cell densities of 2.97–3.14 Log CFU/ml and *P. fluorescens* (2 ml) at cell densities 3.02–3.15 Log CFU/ml were added to sterile distilled water (SDW), buffered peptone water (BPW), and trypticase soy broth (TSB). Bacterial populations suspended in SDW and BPW are referred to as resting cells due to limited nutrient composition in this media. Bacterial cells were grouped as follows: monocultures (*Ec* 43894, 43895, 35150 and *Pf* 2-79) and a mixed combination (co-culture) of *Ec* 43894 and 2-79, *Ec* 43895 and 2-79, *Ec* 35150 and 2-79. Other co-cultures consisted of 43895 + Q8R1, and 35150 + Q287. All bacterial cells were prepared in separate tubes containing SDW, BPW, and TSB. Some representative combinations instead of a complete factorial was used due to incubator space limitation.

2.4. Effects of storage temperatures and time on bacterial populations

To assess the effects of storage temperature on bacterial populations, (*E. coli* O157:H7 strains (3) × *P. fluorescens* strains (3) × storage temperatures (4), with 3 replicates was established. A control treatment in which the 3 strains of *Ec* were stored in BPW as monocultures also was established. A co-culture was constituted by aliquoting 1 ml of *Ec* and adding to 1 ml of *Pf* in the mixture tube prior to storage. All test tubes containing bacterial cell populations

were stored at 5, 10, 15, 20, 25, 35, and 37 °C for 24 h. Periodically (0, 2, 4, 6, and 24 h), aliquots (100 μl) from monocultures and the co-cultures were platted on RFCM medium (*Ec*) and PAF (*Pf*) described above to enumerate colony forming units (CFU/ml). The plates were incubated at 26 °C for 24 h and 37 °C for 48 h which were optimum growth temperatures for *Pf* and *Ec*, respectively. The co-inoculated cultures (mixtures) were quantified on both media and the colonies of *Ec* were enumerated based on their blue-black precipitates on the selective medium as opposed to *Pf*, which had translucent/white colony morphology that fluoresce on PAF medium.

2.5. Ratio of P. fluorescens to E. coli O157:H7 populations

To evaluate if cell density of biocontrol microbe relative to the pathogen (*Ec*) can affect bacteria growth, experiments consisting of 2 storage temperatures (20, 30 °C) × 3 bacterial cell density ratios of high (100×), low (10×), less than equal (<10×) were established The pathogen combinations were *Ec* 35150 + *Pf* 2-79, *Ec* 43894 + *Pf* 2-79, and *Ec* 43895 + *Pf* 2-79. The cell density ratios of *Pf* to *Ec* used in this experiment were based on overnight incubated bacterial suspensions serially diluted to: 10^5 *Pf* and 10^3 *Ec*, 10^4 for *Pf* and 10^3 for *Ec*, and 10^4 *Pf* and 10^5 *Ec* representing high, low and less than equal cell densities of *Pf* relative to *Ec*. In this experiment, all dilutions were made in BPW and the bacterial cells were stored as stated above. Bacterial colonies from monocultures or co-cultures with typical characteristics of *E. coli* O157:H7 were enumerated based on colony morphology on RFCM (Restaino et al., 1999) and the translucent colony morphology and fluoresce of *Pf* on PAF medium (Olanya et al., 2012).

2.6. Statistical analysis

Ec and *Pf* counts from monocultures and mixed cultures were converted to Log_{10} CFU/ml. The average bacteria populations and associated standard errors were computed by Proc Means of the Statistical Analysis System (SAS, SAS Institute, Cary, NC). The effects of temperatures and storage time on populations of *Pf* to *Ec* in monocultures and co-cultures were computed by analysis of variance (ANOVA). Significance of treatment effects were computed by least significance difference (LSD) statistics.

3. Results and discussion

3.1. Effects of incubation temperatures and time on the survival of P. fluorescens (Pf) and E. coli O157:H7 (Ec) populations in monocultures

The initial cell densities of *Ec* inocula used in this experiment were 3.14 ± 0.20, 3.07 ± 0.20, and 2.97 ± 0.19 Log CFU/ml for the strains 43894, 43895, and 35150, respectively. For *Pf* isolates 2-79, Q287, and Q8R1, the initial inocula were 3.02 ± 0.21, 3.15 ± 0.19, and 3.14 ± 0.20 Log CFU/ml, respectively.

When bacteria were stored at 5, 15, 25 and 37 °C for 24 h as monocultures in SDW, *Ec* populations slightly decreased and the bacterial numbers were not significantly different ($P > 0.05$) across all temperatures evaluated (data not presented). Populations of *Pf* isolates similarly treated and stored in SDW showed the same trend. The storage temperatures had no significant effect on the survival of *Ec* and *Pf* populations in SDW monoculture, perhaps due to lack of nutrient substrate for bacterial growth. On BPW monoculture, *Ec* and *Pf* populations varied slightly, and were significantly ($P < 0.05$) greater at 25 and 37 °C than at 5 and 15 °C storage temperatures (Table 1). This is not totally unexpected since there is little substrate or nutrients in SDW or BPW to support bacterial growth in monocultures.

The populations of *Ec* and *Pf* were affected when bacteria were grown in co-cultures in SDW and BPW (Table 2). *Ec* and *Pf* populations were significantly ($P < 0.05$) impacted by storage

Table 1
Effects of storage temperatures on populations of *P. fluorescens* isolates 2-79, Q8R1, Q287 and *Escherichia coli* O157:H7, strains 43894, 43895 and 35150 grown in monocultures in buffered peptone water (BPW) for 24 h.

Growth substrate	Temp (°C)[a]	43894[b] (Log CFU/ml)	43895[b] (Log CFU/ml)	35150[b] (Log CFU/ml)	2-79[c] (Log CFU/ml)	Q8 R1[c] (Log CFU/ml)	Q2 87[c] (Log CFU/ml)
BPW	5	3.46 ± 0.09a	3.15 ± 0.15a	3.57 ± 0.07a	2.54 ± 0.21a	2.84 ± 0.25a	2.75 ± 0.34a
	15	3.51 ± 0.62a	3.46 ± 0.59a	3.00 ± 0.51a	3.21 ± 0.47ab	3.85 ± 0.62a	3.81 ± 0.64a
	25	5.78 ± 0.85b	5.73 ± 0.91b	5.85 ± 0.79b	4.11 ± 0.21b	5.45 ± 0.34b	7.60 ± 0.92b
	37	6.09 ± 0.71b	6.11 ± 0.72b	6.14 ± 0.73b	4.70 ± 0.37b	5.15 ± 0.56b	6.41 ± 0.21b

[a] Temperatures at which monocultures of bacteria were incubated in BPW prior to plating on medium.
[b] *E. coli* O157:H7 strains were plated on R & F *E. coli* O157:H7 chromogenic plating medium.
[c] *P. fluorescens* isolates plated on Pseudomonas Agar F (PAF). Data are the means of 3 replications. Means and associated standard errors in the same column within a growth substrate followed by different letters are significantly different ($P < 0.05$).

Table 2
Effects of storage temperatures on populations of *P. fluorescens* isolates 2-79, Q8R1, Q287 and *Escherichia coli* O157:H7 strains 43894, 43895 and 35150, grown in mixtures in sterile distilled water (SDW), and buffered peptone water (BPW) for 24 h.

Growth substrate	Temp (°C)[a]	43894[b] (Log CFU/ml)	43895[b] (Log CFU/ml)	35150[b] (Log CFU/ml)	2-79[c] (Log CFU/ml)	Q8 R1[c] (Log CFU/ml)	Q2 87[c] (Log CFU/ml)
SDW	5	2.61 ± 0.32a	3.78 ± 0.08ab	2.22 ± 0.24a	2.13 ± 0.24a	2.03 ± 0.12a	3.06 ± 0.10a
	15	2.59 ± 0.29a	3.78 ± 0.06b	2.45 ± 0.21b	1.59 ± 0.16a	1.64 ± 0.05a	2.59 ± 0.05a
	25	1.72 ± 0.17a	3.68 ± 0.07a	2.24 ± 0.24ab	3.19 ± 0.43b	–[d]	1.50 ± 0.20a
	37	3.46 ± 0.03a	2.38 ± 0.19a	–	2.35 ± 0.05a	2.44 ± 0.12a	–
BPW	5	3.35 ± 0.05a	3.16 ± 0.17a	3.49 ± 0.08a	2.59 ± 0.12a	2.89 ± 0.07a	2.98 ± 0.13a
	15	5.21 ± 0.14ab	5.34 ± 0.13ab	5.10 ± 0.14ab	3.96 ± 0.23a	4.92 ± 0.04b	5.35 ± 0.11b
	25	6.43 ± 0.02bc	6.30 ± 0.07bc	6.31 ± 0.03bc	–	7.15 ± 0.17c	6.68 ± 0.09c
	37	8.48 ± 0.03c	8.23 ± 0.18c	8.39 ± 0.05c	–	6.40 ± 0.10c	6.30 ± 0.00c

[a] Temperatures at which monocultures of bacteria were stored in SDW or BPW prior to plating on media.
[b] *E. coli* O157:H7 (Ec) strains were co-cultured with *P. fluorescens* (Pf) as follows: Ec 43894 and Pf 2-79, Ec 43895 and Pf Q8R1, Ec 35150 and Q287, respectively. *E. coli* O157:H7 were plated on R & F *E. coli* O157:H7 chromogenic plating medium.
[c] *P. fluorescens* isolates plated on Pseudomonas Agar F (PAF). Data are the means of 3 replications. Means and associated standard errors in the same column within a growth substrate followed by different letters are significantly different ($P < 0.05$).
[d] Bacterial colonies were too numerous to be counted.

temperatures as bacterial numbers increased significantly at 25 and 37 °C in BPW when compared to 5 °C storage (Table 2). For the co-cultures in SDW, increases in bacterial numbers were relatively lower than in BPW. The pH of substrate was maintained at 7.2 at the start of the experiment which was an optimal pH for normal physiological metabolism of the bacteria. Therefore, the lack of nutrients in SDW, the pH of 7.2 preserved the bacterial cell populations in a resting form. The choice of storage temperatures of 25 and 37 °C was designed to accommodate the temperature optima for growth of *Pf* and *Ec*, respectively. Therefore, the greater bacterial populations at 25 and 37 °C may be explained by temperatures which were conducive for cell growth while in storage relative to 5 or 15 °C. Populations of *Pf* (2-79) in BPW monoculture increased during storage for 24 h and the numbers determined were significantly ($P < 0.05$) different from the populations stored at 0 h (Tables 1 and 2). The implications of the results are that bacterial populations can be best quantified when optimum storage temperatures for growth are utilized.

Variation in the growth kinetics of *E. coli* O157:H7 were reported to be influenced by temperature and pH (Duffy et al., 1999). In this study, no significant differences among strains of *Ec* were observed and we can attribute this effect to the limited nutrient composition in the substrate especially the SDW. In this instance, populations of *Pf* and *Ec* in monocultures at the optimal temperature were similar to those of other storage temperatures due to nutrient deficiency as has been previously documented (McKellar, 2007). On the other hand, populations of *E. coli* O157:H7 (43894) grown in TSB were noted to increase from about 3.4 at 5 °C to 8.7 Log CFU/ml at 37 °C in monocultures, while *P. fluorescens* (2-79) increased to about 6.4 Log CFU/ml at 25 °C, but decreased at 37 °C (Fig 1). This may be attributed to the near optimum temperature of 37 °C conducive for the growth of *E. coli* O157:H7 and of 26 °C for *P. fluorescens*. The difference in our results with that of other researchers may also be attributed to the fact the isolate of *P. fluorescens* used in this study is saprophytic and non-plant pathogenic, while the *P. fluorescens* isolates used in other studies are food spoilage strains with pectinolytic and other enzymes for degradation or food or other substrates.

The monocultures of *Ec* strains 43894, 43895 and 35150 stored at 20 °C had average populations of 1.50, 2.18, and 1.88 Log CFU/ml at the initial (0 h) storage time. After 6 h of storage in BPW, bacterial numbers averaged 2.33, 2.83 and 2.30 Log CFU/ml, respectively (Fig 2). When *Ec* strains were co-cultured with *Pf*, initial populations of *Ec* at 0 h were 1.75, 1.92, and 1.72 Log CFU/ml for 43894, 43895, and 35150, respectively. At 6 h of storage populations of *E. coli* O157:H7 were 2.50, 2.56, and 2.20 Log CFU/ml of BPW, for the three strains; respectively (Fig 2). At 24 h of storage, populations could not be enumerated as colonies were too numerous to quantify. The comparisons of *E. coli* O157:H7 strains in monocultures to populations of *Ec* co-cultured with *Pf* revealed low suppression of *Ec* populations in the average range of 0.20–1.13 Log CFU/ml (Fig 3). This implies that suppressive effects may be limited when CFU of both microbes are high. The presence of nutrients in the substrate and their effect on survival or growth of *E. coli* O157:H7 in co-culture with *P. fluorescens* in trypticase soy broth (TSB) stored at 20 and 35 °C were investigated (Fig 4). Bacterial populations in TSB increased with storage times from 0 to 48 h, when maximum population levels were attained (Fig 4). The monoculture of *Ec* at 0 h had mean population of 2.17 and 2.31 Log CFU/ml for 20 and 35 °C, and for *Ec* in the co-culture, bacterial numbers at 20 and 35 °C were 1.71 and 1.90 Log CFU/ml respectively (Fig 4). The monoculture of *Pf* at 20 and 35 °C at 0 h had mean bacterial numbers of 3.27 and 3.14 Log CFU/ml, while when co-cultured with *Ec*, *Pf* numbers at 20 and 35 °C were 3.09 and 2.88 Log CFU/ml of TSB, respectively. After 48 h of storage, the average maximum bacterial numbers for *Ec* in monoculture and co-culture were 8.75 and 5.83 Log CFU/ml at 20 °C, and at 8.85 and 9.03 Log CFU/ml at 35 °C, respectively (Fig. 4).

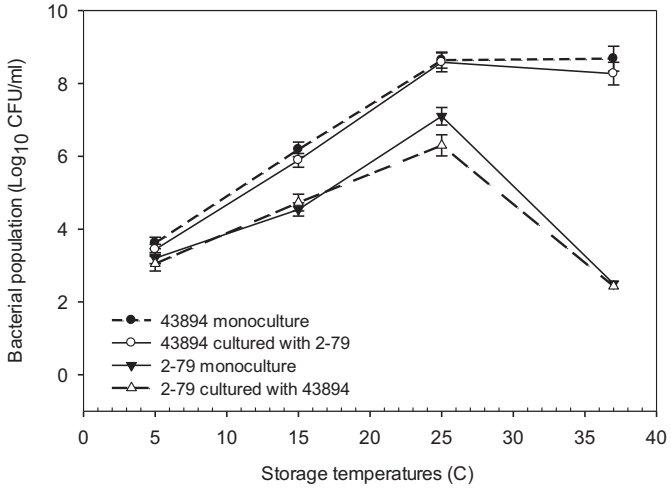

Fig. 1. Effects of storage temperatures (5, 15, 25, and 37 C) on populations of *E. coli* O157:H7 (43894) and *P. fluorescens* (2-79) grown in monocultures and co-cultures on the two bacteria in TSB for 24 h. Means of observations ± standard error of the mean.

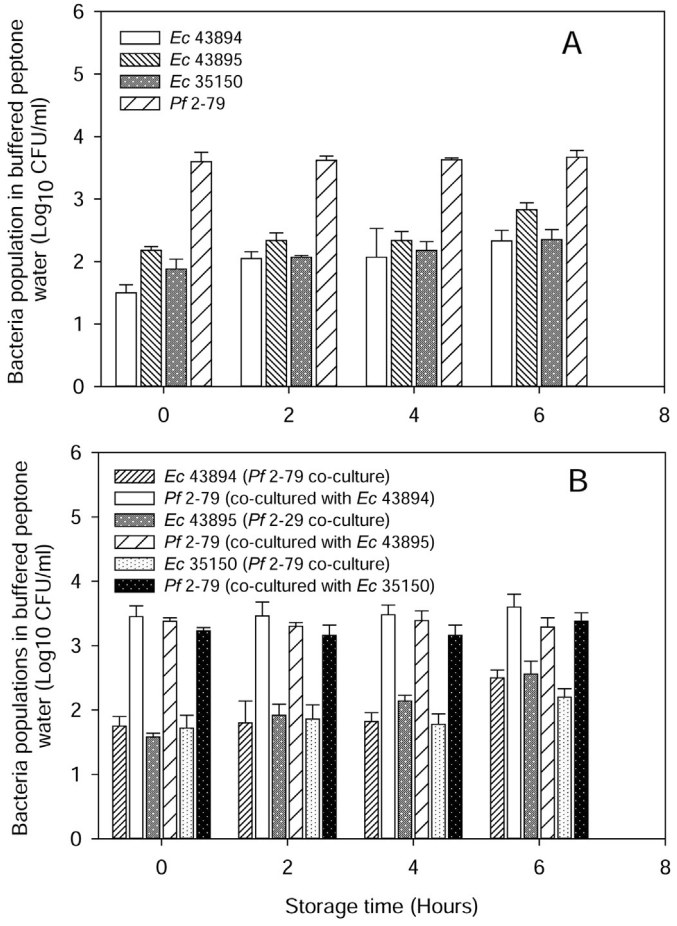

Fig. 2. Effects of storage time on populations of *Escherichia coli* O157:H7 strains 38494, 38495, and 35150 grown in monocultures (A) and co-cultured (B) with *Pseudomonas fluorescens* (2-79). The bacteria in buffered peptone water (BPW) were incubated at 20 °C and bacterial populations quantified at 0, 2, 4, and 6 h of storage. Means of observations ± standard error of the mean.

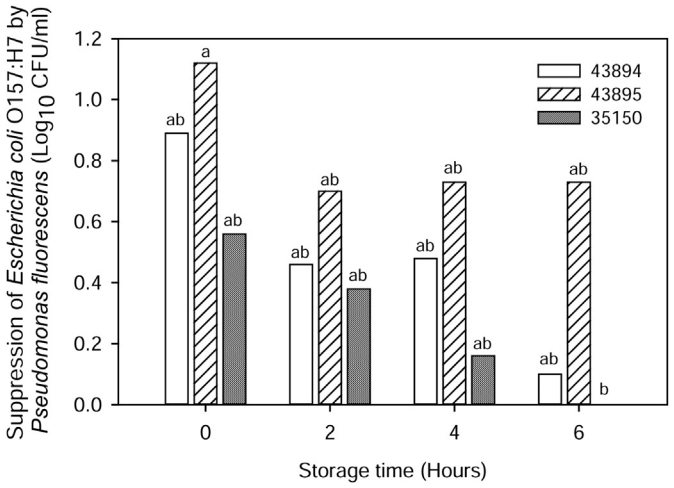

Fig. 3. Effects of co-inoculation of *E. coli* O157:H7 strains with *P. fluorescens* on microbial suppression. Monocultures and co-cultures of *E. coli* O157:H7 and *P. fluorescens* were stored at 20 °C in BPW for 24 h. Suppression was computed as the difference in bacteria log numbers of monoculture of *E. coli* O157:H7 strain relative to the same strain (*E. coli* O157:H7) co-cultured with *P. fluorescens* (2-79) under identical conditions.

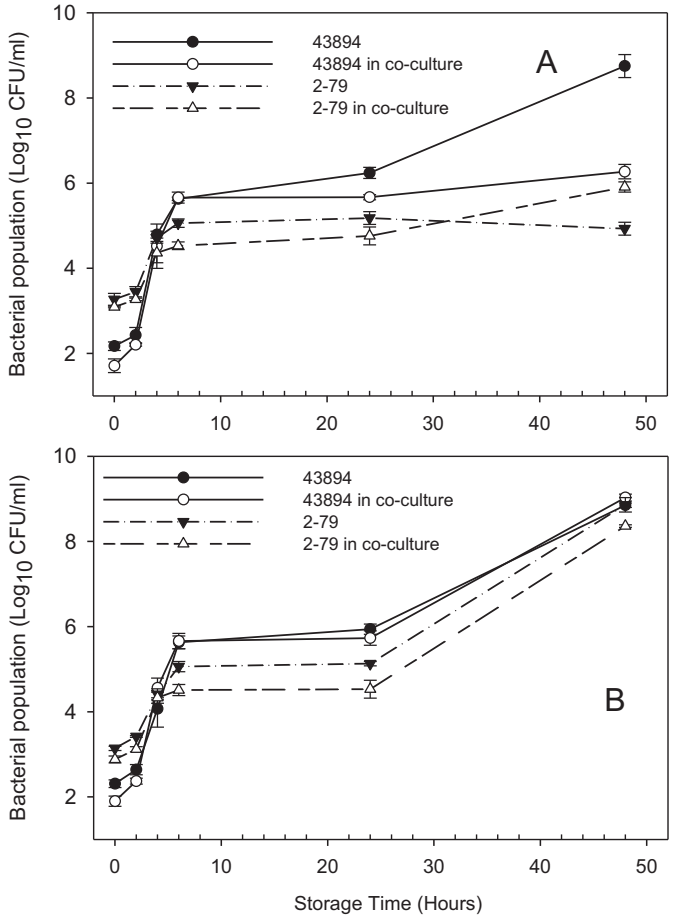

Fig. 4. Effects of storage time on populations of *Escherichia coli* O157:H7 strain 38494 grown in monoculture and co-cultured with *Pseudomonas fluorescens* (2-79). The bacteria in trypticase soy broth (TSB) were incubated at 20 °C (A) and at 35 °C (B) and bacterial populations determined at 0, 2, 4, 6, 24, and 48 h of storage.

In nature, *P. fluorescens* occurs as epiphytic microflora on many food produce, plants, and soil; and have been isolated from various unprocessed vegetables. Populations of *P. fluorescens* of 10^4 cells/cm^2 of foliage of cabbage have been reported (Lamikanra & Iman, 2005). On fresh leafy vegetables, the count of *P. fluorescens* is approximately 10^5 CFU g^{-1} of tissue (Willocx, Mercier, Hendrickx, & Tobback, 1999). However, in soil rhizosphere, bacterial counts in the range of 10^6–10^9 CFU/g of soil have been reported (Weller, 1988). Our choice of 10^3 CFU/ml initial cell populations in monoculture and an approximate level of 10^6 CFU/ml as co-cultures (mixed culture) was within range as reported above.

3.2. Effects of storage temperatures and time on survival of *P. fluorescens* and *E. coli* O157:H7 populations in co-cultures

At 24 h of storage in BPW, the populations of *Ec* strains in co-cultures were lower than those in monocultures of the same substrate (Table 3). In general, the average suppressive values of *Ec* bacterial populations in co-cultures relative to monocultures were: 1.20, 0.62, and 0.37 Log CFU/ml of BPW. The suppressive values did not always decrease with increase in storage time but varied across time and among bacterial isolates combinations tested. For example, there was very low suppression level of *Ec* 43894 by *Pf* 2-79 at 37 °C based on the storage temperature studies (Fig. 1), suggesting that low antagonistic microbial activity or low competition may occur at 37 °C. On the other hand, the suppressive effects were greatest at 25 °C when 2-79 was co-cultured with 43894 relative to 2-79 alone. Therefore, the levels of suppression of *Ec* by *Pf* may vary with storage temperatures when cultured in-vitro and may be impacted particularly if the substrate has abundant nutrients. When trypticase soy broth (TSB) was used, the suppression of *Ec* growth at 20 °C generally ranged from 0.24 to 2.87 Log CFU/ml compared to 0.21 to 0.48 Log CFU/ml at 35 °C (Fig 4). The results indicate higher suppression of *Ec* in broth media than BPW and this variation in suppression may be attributed to competition for nutrients. Therefore, suppressive effects of the biocontrol microbe can best be quantified in nutrient abundant substrate.

Several reports in the literature have documented that *P. fluorescens* can inhibit other pathogenic bacteria due to competition for substrates or utilization of specific growth compounds such as siderophores (Buchanan & Bagi, 1999; Cheng et al., 1995; Farrag & Martin, 1989; Freedman, Kondo, & Willrett, 1989). On the contrary, in other studies, it has been suggested that *Pf* could also stimulate the growth of other microbes or have no detrimental effect (Quinto, Franco, Fente, Vazquez, & Cepeda, 1997). In our study, low inhibition levels were detected, and the difference between our finding and the others could also be explained by the fact that in some of the above research, the bacterial strains used were different from those in our study in which the strains were non-pectinolytic, and non-plant pathogenic.

3.3. Influence of Pf to Ec cell density ratios on bacteria survival in co-culture

The influence of *Pf* to *Ec* cell density ratios upon survival in co-cultured media stored at 20° and 30 °C were investigated. The ratio of *P. fluorescens* to *E. coli* O157:H7 cells had variable effects on bacterial populations in co-cultures. At 20 °C, a cell density ratio of 100× resulted in significantly greater ($P < 0.05$) populations of *Ec* strain 43894 than at the other ratios (Table 4). For *P. fluorescens*, a significant difference ($P < 0.05$) in bacterial numbers was recorded when *Ec* strain 35150 was co-cultured with *P. fluorescens*, 2-79 (Table 4). At 30 °C, no significant ($P > 0.05$) differences in populations were detected for *E. coli* O157:H7 strains irrespective of the cell density ratios. However, *P. fluorescens*, co-inoculated with *Ec*

Table 3
Effects of storage time on populations of *Escherichia coli* O157:H7 strains 43894, 43895 and 35150 and *P. fluorescens* isolates 2-79, Q8R1, Q287 in co-culture (mixtures) and monocultures incubated at 10 °C in buffered peptone water (BPW) at different time intervals.

Storage time (hours)[a]	2-79 Mono-culture[b] (Log CFU/ml)	43894 Mono-culture[c] (Log CFU/ml)	43894 Co-culture with 2-79[d] (Log CFU/ml)	2-79 Mono-culture[b] (Log CFU/ml)	43895 Mono-culture[c] (Log CFU/ml)	43895 Co-culture with 2-79[d] (Log CFU/ml)	2-79 Mono-culture[b] (Log CFU/ml)	35150 Mono-culture[c] (Log CFU/ml)	35150 Co-culture with 2-79[d] (Log CFU/ml)
0	2.41 ± 0.11bc	1.99 ± 0.09ab	1.10 ± 0.17a	2.09 ± 0.12b	2.65 ± 0.13a	1.53 ± 0.21b	2.10 ± 0.18b	1.56 ± 0.24a	1.00 ± 0.00a
2	2.24 ± 0.06c	1.85 ± 0.48b	1.39 ± 0.12a	1.97 ± 0.14b	2.20 ± 0.16a	1.50 ± 0.28b	2.13 ± 0.15b	1.68 ± 0.17a	1.30 ± 0.00a
4	2.36 ± 0.04bc	1.87 ± 0.11b	1.41 ± 0.09a	2.20 ± 0.17b	2.12 ± 0.17a	1.39 ± 0.43b	1.98 ± 0.18b	1.36 ± 0.10a	1.20 ± 0.17a
6	2.48 ± 0.11bc	1.20 ± 0.10b	1.10 ± 0.17a	2.30 ± 0.02b	2.21 ± 0.04a	1.48 ± 0.00b	2.31 ± 0.03b	1.38 ± 0.81a	–[e]
24	3.32 ± 0.04ab	1.88 ± 0.03b	1.00 ± 0.21a	3.31 ± 0.06a	1.52 ± 0.24b	–[e]	3.00 ± 0.06ab	1.39 ± 0.58a	1.20 ± 0.08a
48	3.70 ± 0.00a	2.46 ± 0.04a	1.26 ± 0.24a	3.64 ± 0.04a	3.03 ± 0.16a	2.41 ± 0.11a	3.62 ± 0.00a	1.83 ± 0.46a	1.46 ± 0.28a

[a] Time intervals at which monocultures and mixed cultures of *Escherichia coli* O157:H7 and *P. fluorescens* were sampled and plated on R & F *E. coli* O157:H7 chromogenic medium and Pseudomonas Agar F (PAF), respectively. Data are the means of 3 replications. Means and associated standard errors in the same column within a growth substrate followed by the different letters are significantly different ($P < 0.05$).
[b] *P. fluorescens* isolate 2-79 (non-pathogenic and non-pectinolytic) was incubated alone in BPW tubes as separate controls.
[c] Each strain of *E. coli* O157:H7 (43894, 43895, 35150) was incubated and plated alone (monoculture).
[d] Co-culture with *P. fluorescens* (2-79).
[e] Bacterial numbers were too numerous to count.

Table 4
Effect of *Pseudomonas fluorescens* (2-79) cell density relative to *Escherichia coli* O157:H7 strains 35150, 43894, 43895 co-cultured in buffered peptone water (BPW) on bacterial populations.

Temp[a]		20 °C[d]		30°C[d]	
Cell density Ratios[b]	Bacteria co-culture[c]	*E. coli* O157:H7 (Log CFU/ml)[c]	*P. fluorescens* (Log CFU/ml)[e]	*E. coli* O157:H7 (Log CFU/ml)[c]	*P. fluorescens* (Log CFU/ml)[e]
100×	35150 + 2-79	1.35 ± 0.49a	2.87 ± 0.01a	2.93 ± 0.02a	3.66 ± 0.00a
10×	35150 + 2-79	1.59 ± 0.11a	1.65 ± 0.07b	3.01 ± 0.03a	2.63 ± 0.16b
<10×	35150 + 2-79	1.48 ± 0.00a	–[f]	2.97 ± 0.07a	1.46 ± 0.41c
100×	43894 + 2-79	3.32 ± 0.04a	1.00 ± 0.00a	2.92 ± 0.03a	2.67 ± 0.00a
10×	43894 + 2-79	1.69 ± 0.27b	1.70 ± 0.00a	2.93 ± 0.02a	2.53 ± 0.19a
<10×	43894 + 2-79	1.77 ± 0.07b	–	2.78 ± 0.35a	1.69 ± 0.09a
100×	43895 + 2-79	1.92 ± 0.13a	2.67 ± 0.11a	2.49 ± 0.14a	3.66 ± 0.00a
10×	43895 + 2-79	2.00 ± 0.26a	1.84 ± 0.09a	2.43 ± 0.18a	2.15 ± 0.27b
<10×	43895 + 2-79	2.01 ± 0.15a	–	2.75 ± 0.06a	1.69 ± 0.98b

[a] Temperatures at which bacterial suspensions were incubated in BPW for 24 h.
[b] The 100× and 10× refers to hundred-fold, ten-fold difference in dilution of *P. fluorescens* from overnight cultures relative to *Ec.* The <10× indicates that initial *E. coli* O157:H7 was ten times greater than *P. fluorescens* inoculum.
[c] Strains of *E. coli* O157:H7 in co-culture with *P. fluorescens* (2-79).
[d] The initial mean cell density (inoculum) of *E. coli* O157:H7 strains 35150, 43894, and 43895 were 3.19, 2.99, and 3.27 Log CFU/ml of BPW, and 2.97 Log CFU/ml for *P. fluorescens* (2-79) at 20 °C. At 30 °C, the initial mean cell density (inoculum) of *E. coli* O157:H7 strains 35150, 43894, and 43895 were 3.60, 3.65, and 2.93 Log CFU/ml of BPW, and 3.61 Log CFU/ml for *P. fluorescens* (2-79).
[e] Data was averaged across 3 replications. Means and associated standard errors followed by the different letters are significantly different ($P < 0.05$).
[f] No counts of *P. fluorescens* was recorded.

strains 43895 and 35150 had differing population levels with decreasing ratio as expected. When the cell ratios of *P. fluorescens* relative to *E. coli* O157:H7 were identical, and samples stored in SDW for 24 h, the bacterial numbers did not differ significantly among storage temperatures of 5, 10, 25, and 37 °C. The populations of *E. coli* O157:H7 in monocultures relative to that in co-culture at the three ratios were also not statistically different ($P > 0.05$).

3.4. Conclusions

The results of this research suggest that cell growth of *E. coli* O157:H7 in the presence of *P. fluorescens* (non-pectinolytic and non-plant pathogenic) may be impacted by nutrient availability. Although low to moderate levels of suppression of *E. coli* O157:H7 by *P. fluorescens* were recorded when both bacteria were incubated in co-cultures, the suppressive levels varied with pathogen-strain combinations, storage times, and nutrient availability (BPW vs TSB). The biocontrol implications of the experiments suggest that *P. fluorescens* co-cultured with *E. coli* O157:H7 under controlled, laboratory conditions can be used to assess interactions and suppressive effects of bacteria on nutrient-rich substrate to enhance food safety. Under nutrient deficient conditions such as SDW or BPW, suppressive effects of *P. fluorescens* cannot be optimally quantified. The survival of *P. fluorescens* and *E. coli* O157:H7 in co-cultures can be utilized to assess the biocontrol potential of *P. fluorescens* in reducing contamination of *E. coli* O157:H7 on produce especially during transitory temperature abuse conditions, such as transportation of leafy greens from fields to packing shades or processing facilities. Although the suppression of *E. coli* O157:H7 by *P. fluorescens* may be a viable option for reducing food contamination, its potential should be assessed on produce to optimize biocontrol efficacy.

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